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Research Paper

Gamisasangja-tang suppresses pruritus and atopic skin inflammation in the NC/Nga murine model of atopic dermatitis

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ABSTRACT

Ethnopharmacological relevance: Gamisasangja-tang (GST) is a traditional herbal formula prescribed for patients with intractable pruritus in association with various inflammatory skin diseases. To evaluate the effects of GST on pruritic skin inflammation and investigate its cellular and molecular mechanisms.
Materials and methods: We orally administered GST to NC/Nga (NC) mice, an animal model of atopic dermatitis. Scratching frequency and the dermatitis index were evaluated, and histological examination was performed using hematoxylin and eosin and toluidine blue staining. The levels of interleukin (IL)-31 and T-helper cell type 2 (T_H2) cytokines were determined in both the dorsal skin and cultured splenocytes by real-time polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. The serum levels of chemokines and immunoglobulin E (IgE) were determined by ELISA. Changes in the inflammatory cell population were analyzed by a hemocytometer.
Results: GST significantly lowered scratching frequency and inhibited increases in dermatitis index, thickness of epidermis/dermis and infiltration of chemokine (C-C motif) receptor 3 $(CCR3)^+$ and cluster of differentiation $(CD)117^+$/FcεRIα (Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide)$^+$ cells in atopic skin. Both *IL*-31 mRNA expression and production were significantly reduced by GST, which was accomrease in the levels of *IL-4*, *IL-5*, and *IL-13*. Further, GST treatment suppressed the secretion of eotaxin, TARC (thymus and activation-regulated chemokine), IgE, and increases in the number of basophils and eosinophils in the blood.
Conclusion: GST may have potential as an effective treatment for pruritic skin disease such as atopic dermatitis.

1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease characterized by pruritic eczematous lesions. It is currently accepted that the development of atopic skin lesions is associated with skin barrier dysfunction and a skewed balance of T-helper type 2 (T_H2) cells in the immunological system (Berke et al., 2012). Pruritus is not only the most distressing symptom in patients with AD, but is also a major cause of skin barrier dysfunction (Kim et al., 2006). Cutaneous abnormalities such as dryness may initiate an itching sensation that leads to mechanical injury form scratching, and a recurrent itching-scratching cycle can worsen the disease by increasing the release of proinflammatory cytokines and chemokine production (Kabashima, 2013). This inflammatory process subsequently directs the

Abbreviations: ANOVA, analysis of variance; CCR3, chemokine (C-C motif) receptor 3; CD, cluster of differentiation; DNCB, 2,4-dinitrochlorobenzene; DNFB, 2,4-dinitrofluorobenzene; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; FcεRIα, Fc fragment of IgE, high affinity I, receptor for, alpha polypeptide; GST, Gamisasangja-tang; Dex, dexamethasone; IgE, immunoglobulin E; IL, interleukin; NC, NC/Nga; PBS, phosphate buffered saline; PE, phycoerythrin; PerCP, peridinin chlorophyll protein complex; PCR, polymerase chain reaction; ST, sasangja-tang; TARC, thymus and activation regulated chemokine; Th2, T-helper cell type 2

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recruitment of pathogenic leukocytes to the skin (Ley, 1996), and thus, itching directly contributes to the development of eczematous skin lesions. Recently, interleukin (IL)-31, originally identified as a T_H2 cytokine, has been proposed as a pruritogenic cytokine (Cornelissen et al., 2012). IL-31 transgenic mice develop spontaneous severe pruritus and defects in skin barrier formation with trans-epidermal water loss (Dillon et al., 2004). Indeed, skin biopsies of AD patients display increased *IL-31* mRNA expression compared to normal skin, and increased serum levels of IL-31 are correlated with itching and AD severity in human (Raap et al., 2008). Furthermore, the amplification of atopic skin inflammation has been associated with a T_H2 phenotype that includes T_H2 cytokine secretion, blood eosinophilia, and increased serum immunoglobulin E (IgE) (Akdis et al., 2000). It has been well known that increased levels of T_H2 cytokine expression, including IL-4, IL-5, and IL-13, are correlated with the severity of atopic dermatitis (Leung et al., 2004). IL-4 and IL-13, which are produced by both basophils and T_H2 cells, together direct IgE switching in B cells (Sallusto et al., 1998), and IL-5 promotes the survival and activation of eosinophils, resulting in eosinophilia and infiltration of the cells into skin (Simon et al., 2004). Chemokines such as eotaxin and thymus and activation-regulated chemokine (TARC), which is produced by T_H2 cells, keratinocytes, or endothelial cells, contribute to the infiltration of leukocytes including eosinophils, monocytes, and mast cells (Homey et al., 2006). The serum levels of IgE are also associated with the severity of AD (Matsuda et al., 1997), and the cross-linking of FCεRI (Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide) on the mast cell surface with IgE and antigens generates prompt release of various inflammatory mediators, leading to atopic skin inflammation, itching, and erythematous skin lesions (Novak et al., 2003).

Gamisasangja-tang (GST) consists of six herbs including *Stemonae radix, Spirodelae herba, Cnidii fructus, Sophora flavescens root, Angelica gigas root, and Clematidis radix.* Sasangja-tang (ST), also called She-chuang-zi-tang, is described in the traditional herbal medicine textbook *Wai-ke-zheng-zong* and has been used for centuries in the treatment of inflammatory skin disease(Chen, 1983). ST was modified by adding *S. radix* and *S. herba* to formulate GST to improve therapeutic effects; because these two herbs were empirically recognized as an effective treatment for itching by Korean medical practitioners. Protostemonamide, stenonamine, and stenonine are stemona alkaloids, which are the main components from *S. radix* belonging to the Stemonaceae family (Yang et al., 2009). *Spirodela polyrrhiza* (Lemnaceae) herba had shown to contain several kinds of flavonoids such as vitexin, orientin, apigenin, and luteolin (Qiao et al., 2011; Seo et al., 2012). Torilin, torilolone, and osthol are the main active compounds found in *Cnidii monnieri* (Umbelliferae) fruit (Basnet et al., 2001; Oh et al., 2002). Matrine and oxymatirne are the most well-known bioactive alkaloids isolated from the root of *S. flavescens* (Leguminosae) family (Lin et al., 2011; Yang et al., 2014). The major constituents of *A. gigas* (Umbelliferae) root include wide variety decursinol derivatives such as decursin, decursidin, and decursinol (Kang and Kim, 2007). Saponin and clematernoside were discovered as chemical constituents from the root part of *Clematidis terniflora* (Ranunculaceae) (Kawata et al., 1998).

To evaluate the therapeutic effects of GST on atopic dermatitis-like skin lesions and to provide scientific evidence for its anti-pruritus effects, we utilized the NC/Nga (NC) mouse, a murine atopic dermatitis model. These animals develop clinical signs of AD that begin with scratching behavior followed by the onset of eczematous skin lesions (Matsuda et al., 1997). Here we report that oral administration of GST significantly suppresses itching, which could be mediated by the suppression of IL-31 expression. Further, GST treatment led to reductions in the thickness of the epidermis and dermis and in the degree of inflammatory cell infiltration. These changes could be mediated by the inhibitory effects of GST on various pathogenic factors such as IgE and TARC as well as T_H2 cytokines.

2. Materials and methods

2.1. Preparation of GST

The herbs, *S. radix* (*Stemona sessilifolia* (Miq.) Miq.), *S. herba* (*S. polyrrhiza* (L.) Schleid.), *C. fructus* (*Cnidium monnieri* (L.) Cusson), *S. flavescens* root (*S. flavescens* Aiton), *A. gigas root* (*A. gigas* Nakai), and *C. radix* (*C. terniflora* var. *manshurica* (Rupr.) Ohwi) were purchased from Dong Kyung Pharm Co., Ltd. (Seoul, Korea). The herb samples were identified by Professor C.G. Son (College of Oriental Medicine, Daejeon University, Daejeon, Korea). Voucher specimens (No. 2014-004 to 009) of the collected herb samples were deposited in the herbarium, according to the procedure described by Park (Park et al., 2014b). GST was obtained by boiling the six herbs (1:1:1:1:0.5:0.5) in distilled water at 100 °C for 2 h. The boiled herbs were then filtered through a Whatman no. 2 filter (Maidstone, UK), concentrated under vacuum conditions, and freeze-dried. The yield of the dried extract was approximately 10.3%. The extract was stored at −80 °C and dissolved in phosphate-buffered saline (PBS) before use.

2.2. Standardization of GST

To standardize GST, chlorogenic acid, vitexin, decursin, and torilin were used as surrogate markers and the content of each was quantified in GST samples (Ahn et al., 1996; Cho et al., 2008; Ge et al., 2007; Kim et al., 2010). Using high-performance liquid chromatography (HPLC), standardization of GST was carried out on a Waters system (Waters Corp., Milford, MA, USA), consisting of separation module (e2695) with a photodiode array detector (2998). UV absorbance was monitored from 200 to 500 nm. Qualitative analysis was carried out at 254 nm at a column temperature of 40 °C. Separation was carried out using an YMC-Triart C18 (250 × 4.6 mm; particle size, 5 μm; YMC Co. Ltd., Japan). The mobile phase was composed of 1% acetic acid in water (v/v, solvent A) and acetonitrile (solvent B). The flow rate was 1 mL/min. The gradient was 0.0 min, 90% A; 30.0 min, 60% A; 34.0 min, 25% A; 44.0 min, 10% A; 50.0 min, 10% A. Re-equilibration time was 10 min. The data shows a chromatogram obtained from a standard mixture and GST. The linearity detection of each compound was calculated from three concentrations. The content of each surrogate compound in GST and retention time was indicated (Supplementary data 1).

2.3. Animal experiments

Specific pathogen-free 6-week-old male NC/Nga (NC) mice were purchased from Central Lab Animal Inc. (Seoul, Korea). Animal experiments complied with the guidelines of the Daejeon University Animal Care and Use Committee (Written approval number DJUARB2013-006). The mice were acclimated for 1 week prior to the induction of AD. To induce AD-like skin inflammation, 0.2% 2,4-dinitrochlorobenzene (DNCB, Sigma-Aldrich, St. Louis, MO, USA) dissolved in an acetone and olive oil solution (3:1) was applied once a week on shaved dorsal skin. Treated mice were divided into five groups ($n = 10–12$ per group), and were orally treated with PBS, dexamethasone (Dex, 3 mg/kg/day, Sigma-Aldrich), or GST (100, 250, or 500 mg/kg/day) every day for 5 weeks. The Naïve group was treated with vehicle (acetone and olive oil) on the dorsal skin and was given oral doses of PBS. The severity of dermatitis was assessed once a week by three persons unaware of the identities of the groups, according to the method described by Leung (Leung et al., 1990). A total clinical index of dermatitis severity was defined as the sum of the individual scores graded as follows: 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five signs and symptoms (erythema/hemorrhage,